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Isolation of a Fibronectin-like Molecule from a Marine Bivalve, *Pinctada fucata*, and Its Secretion by Amebocytes

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ABSTRACT—A gelatin-binding protein (GBP) was isolated from the hemolymph of the pearl oyster, *Pinctada fucata,* by affinity chromatography using gelatin-Sepharose. The purified product was a monomer of 220 kDa that showed weak cell spreading activity toward baby hamster kidney cells. GBP was also detected in connective tissues in an insoluble form polymerized by disulfide bonds. Thus, GBP seems to be a fibronectin-like molecule, sharing some properties with vertebrate fibronectin except for the subunit structure of the soluble form. Amebocytes secreted GBP as a component of the extracellular matrix (ECM) *in vitro*, and *in vivo* GBP was deposited together with type I-like collagen in the amebocyte sheath formed at wound sites.

It is known that amebocytes of the pearl oyster synthesize an ECM containing collagen and proteoglycans upon which epithelial cells migrate at wound sites. Therefore, GBP may play a role in the healing process as a cell-adhesion molecule.

INTRODUCTION

Cell-cell and cell-extracellular matrix (ECM) interactions play crucial roles during wound healing. Experimental wounding can serve as a good model for investigating the functions of cells and molecules correlated with morphogenesis. When molluscs are experimentally wounded, amebocytes synthesize ECM at the wound site on which the epidermis is regenerated [1–4]. It has been demonstrated in the pearl oyster, *Pinctada fucata,* that agranular amebocytes (hemocytes) can secrete collagen and proteoglycans as components of the ECM and that epithelial cells migrate on the new ECM at wound sites [5]. In the cockroach, *Leucophaea maderae,* hemocytes secrete a protein that mediates the outgrowth of epithelial cells [6–8]. It has been hypothesized that amebocytes secrete some type(s) of cell-adhesive molecule which help epithelial cells adhere to the substratum. At present, it is unknown what kind of cell-adhesive molecule may be present in invertebrate wound healing.

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Fibronectin, a major cell-adhesive protein, forms a complex with fibrin and mediates the adhesion of epithelial cells as a provisional matrix for skin wound healing in mammals [9]. In corneal healing, fibronectin appears at wound sites after damage [10]. Fibronectin is a macromolecular glycoprotein which binds a number of ligands including cells, collagens, fibrin and proteoglycans [11]. In addition to the role in healing, fibronectin is important in various morphogenetic processes such as embryogenesis, for its binding ability [12–14].

There are substances immunologically related to mammalian fibronectin in a wide range of invertebrates [15]. Fibronectin(-like) molecules have been isolated from the body fluids and somatic tissues of various invertebrates [16–19]. It is involved in cell aggregation in the sponge [20], migration of the primary mesenchyme cells in the sea-urchin embryo [21–22] and gastrulation in *Drosophila* [18]. Thus, fibronectin(-like) molecules occur widely in invertebrates and may correlate with invertebrate wound healing.

To better understand the wound healing system of bivalves, a fibronectin-like substance was isolated from the pearl oyster and the ability of

amebocytes to secrete this substance was studied.

MATERIALS AND METHODS

Purification

Hemolymph was collected from pearl oysters, *Pinctada fucata,* as previously described [23]. Phenylmethylsulfonyl fluoride (PMSF) and EDTA were immediately added to the hemolymph to give a final concentration of 0.1 mM and 5 mM, respectively.

Gelatin-binding protein (GBP) was purified from the hemolymph using a slightly modified procedure for mammalian plasma fibronectin developed by Ruoslahti *et al.* [24]. The batch method was used to adsorb and elute GBP from the gelatin-column. In brief, ten ml of fresh hemolymph was passed through a Sepharose 4B (Pharmacia) column, and the flow through fractions were pooled and then gently agitated with 5 ml of gelatin-Sepharose 4B (porcine skin; Pharmacia) in a plastic tube for 1 hr. The gel was then placed into a column (4×10 cm) and first washed with 50 mM Tris/HCl buffer, pH 7.5 containing 0.5 M NaCl, followed with phosphate buffered saline (PBS). The retained protein was recovered from the gel with 3 ml of 4 M urea in 50 mM Tris/HCl, pH 7.5. All procedures were performed at 4°C.

Protein content was determined using the Bio-Rad Protein Assay.

Cell spreading assay

Affinity-purified GBP was dialyzed exhaustively against PBS. Bovine fibronectin (20 μg/ml; Nitta Gelatin Co.,) or GBP (20 μg/ml) of 200 μl was placed in plastic dishes (24 well plate; Nunc) and incubated for 1 hr at 37°C. After washing the dishes with PBS, a suspension (200 μl) of baby hamster kidney (BHK) cells in Basal Eagle's Medium (10^5 cells/ml) was added and incubated at 37°C for 1 hr. After incubation, the ratio of cells spread on the surface was counted.

Antisera

An aliquat of affinity-purified GBP was emulsified with the same volume of Freund's complete adjuvant, and subcutaneously injected into the back of a rabbit four times at weekly intervals. One week after the final injection, blood was collected from the ear vein and allowed to clot, after which the serum (anti-GBP) was removed after centrifugation at 500×g.

Rabbit anti-human fibronectin and rabbit anti-bovine fibronectin were purchased from Wako Chemical Ltd. and UCB Bioproducts, respectively. Rabbit anti-type I-like collagen of the pearl oyster (anti-collagen) was prepared as previously reported [5].

Electrophoresis and immunoblotting

Procedures as for placenta fibronectin [25] were used to solubilize tissue GBP which was then subjected to immunoblot analysis. In brief, tissue was homogenized with 0.1 M NaCl in buffer A (50 mM Tris/HCl buffer, pH 7.5, 1 mM PMSF, 1 mM EDTA, 20 mM ε-aminocaproic acid, 0.02% NaN_3, 5 μg/ml pepstatin, 5 μg/aprotinin). The tissue homogenate was centrifuged at 20,000×g for 10 min and the precipitate was resuspended in fresh buffer A. This procedure was repeated four times. The precipitate was resuspended in 0.1 M NaCl in buffer A, mixed with urea to give final concentration of 4 M and stirred overnight at 4°C. The mixture was centrifuged, and the supernatant was collected. This is referred to a 4 M urea extract.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [26]. Molecular weights were estimated using Bio-Rad high molecular weight standards and thyroglobulin (Pharmacia). Proteins in the gels were stained with Coomassie Brilliant Blue R-250 or by a silver staining kit (Wako Chemical Ltd.).

A western blot [27] of the proteins was performed at 2 mA/cm^2 for 1 hr. Proteins were detected by immunostaining. Anti-GBP, anti-human fibronectin and bovine fibronectin were used at a dilution of 1:1000. Horseradish peroxidase (HRP)-conjugated swine anti-rabbit immunoglobulins (HRP anti-rabbit Ig; Dakopatts) was used at a dilution of 1:500. Chloronaphthol-H_2O_2 solution (3 mg 4-chloro 2-naphthol in 1 ml ethanol, 5 ml Tris-buffered saline, pH 7.4, and 1 μl 3% H_2O_2) was used as the HRP substrate.

Immunohistochemistry

For the immunohistochemical detection of GBP in the pearl oyster, specimen was dissected and fixed in Bouin's fixative at 4°C for 6 hr. After washing in PBS three times for 1 hr each, the samples were embedded in paraffin and sliced into 5 μm sections. After removing paraffin, the sections were incubated with 5% BSA, then with anti-GBP or normal rabbit serum (1 : 100 dilution with PBS) for 20 min. The sections were then washed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit Ig (FITC anti-rabbit Ig; Dakopatts) at a dilution of 1 : 100.

To detect GBP and type-I like collagen in amebocytes, blood was dropped onto glass slides which were then maintained for 20 min at room temperature to help amebocytes adhere. Cells were fixed with 5% paraformaldehyde in PBS for 20 min and treated with acetone. Samples were treated with anti-GBP or anti-collagen and then FITC anti-rabbit Ig as described.

To observe GBP in the ECM synthesized *in vitro*, amebocytes were cultured for 6 days and fixed as previously reported [5]. The samples were incubated with anti-GBP (1 : 100) for 20 min, washed and incubated with HRP anti-rabbit Ig (1 : 100) for 20 min. Diaminobenzidine (DAB)-H_2O_2 solution (1 mg 3,3′-diaminobenzidine in 5 ml of 50 mM Tris/HCl buffer, pH 7.6, and 5 μl 5% H_2O_2) was used as the substrate for HRP.

In the wound experiment, a shell bead, 7 mm diameter, was implanted into the gonad of a pearl oyster by means of an incision as previously reported [5]. The gonad was dissected from the specimen 7 days after the operation and processed for indirect immunofluorescence as described.

RESULTS

Purification of GBP

Pearl oyster hemolymph was passed through a column of Sepharose 4B, and the flow through fractions were applied to an affinity column with gelatin-Sepharose using the batch method. The retained proteins were recovered from the gel with a small volume of buffered 4 M urea. The eluate gave a homogeneous protein band at the position of 220 kDa in SDS-PAGE (Fig. 1a, b). A subsequent elution of the column with 8 M urea did not yield any detectable proteins. When Sepharose coupled with bovine serum albumin was used for affinity chromatography instead of gelatin-Sepharose, the 220 kDa polypeptide was not obtained. Therefore, the adsorption of the molecule on the gelatin-Sepharose in probably due to specific binding with gelatin. Thus, we refer to the substance as gelatin binding protein (GBP). Approximately 20 μg of GBP was isolated from 10 ml of hemolymph containing 0.9–1.2 mg protein/ml.

Molecular structure

Affinity-purified GBP gave a molecular mass of 220 kDa in the SDS-PAGE under reducing and non-reducing conditions (Fig. 1a, b). These results suggest that GBP is a 220 kDa monomer of polypeptide in the hemolymph.

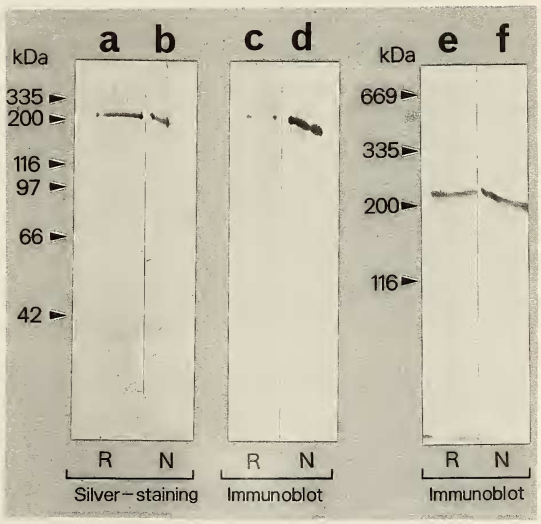


FIG. 1. SDS-PAGE and immunoblot analyses of gelatin-binding protein (GBP) purified from pearl oyster hemolymph. Affinity-purified GBP using gelatin-Sepharose was electrophoresed using 7.5% (a-d) or 5.0% (e-f) polyacrylamide gel and subjected to protein-staining or western blotting. a and b: silver-stained gel. c-f: nitrocellulose sheet immunostained using anti-GBP. In each lane, 0.14 μg of protein was run. R, reduced by 2-mercaptoethanol; N, non-reduced.

Cell-spreading activity

The cell-spreading activity of GBP was studied using BHK cells. Some of the cells elongated on the GBP-coated dish (Fig. 2b). However, the degree of spreading was incomplete as compared with the cells on the bovine fibronectin-coated dish which were fully flattened (Fig. 2c). When partially spread cells were included, the ratio of spreading cells was 16% higher on the GBP-coated than on the non-coated dish (Table 1).

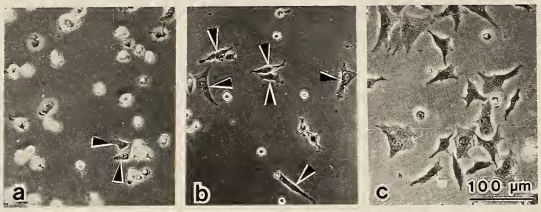


FIG. 2. Effect of GBP on the spreading of BHK cells. BHK cells were incubated for 1 hr on non-coated (a), GBP-coated (b) and bovine fibronectin-coated (c) dishes.

TABLE 1. Ratio of BHK cells spread on non-coated, GBP-coated and bovine fibronectin-coated dish.

Coating	Cells spread on dish / Total cells ×100
None	8*
GBP	24*
Bovine fibronectin	94

* Cells as arrowed in Fig. 2a, b were included.

Localization

In the immunoblot analysis using 7.5% polyacrylamide gel, anti-GBP recognized a 220 kDa polypeptide under non-reducing conditions, but the staining intensity was weak under reducing conditions (Fig. 1c, d). By using a 5.0% gel a protein was recognized by anti-GBP under both conditions (Fig. 1e, f).

Tissue localization of GBP was investigated by indirect immunofluorescence. Fluorescent staining was noted at the following connective tissues; those under the epithelium in the byssus gland, body wall and kidneys (Fig. 3b, d, f); endomysium of the muscle (Fig. 3h); and loose connective tissue distributed between the digestive diverticula and the body wall (Fig. 3j). In particular, intense staining was observed adjacent to the basement membrane of the epithelium in the byssus gland and body wall. No fluorescence was detected in tissues when normal rabbit serum was used as a control.

The 4 M urea extract (adductor muscles tissue) was immunoblotted using 5% polyacrylamide gel to determine the structure of tissue GBP. The adductor muscle was chosen for study because of its immunohistochemical reactivity with anti-GBP and the availability of large quantities. Under reducing conditions, anti-GBP produced an intense band at the 220 kDa position (Fig. 4c). Under non-reducing conditions, a weakly stained band was detected at the position of approximately 450 kDa (Fig. 4d), suggesting that the 220 kDa polypeptide was dimerized or more highly polymerized in the tissue. These bands were not visualized when using Coomassie Brilliant Blue for protein staining (Fig. 4a, b), probably because the concentration was below a detectable level.

Immunological properties

Immunological cross-reactivity of GBP with mammalian fibronectins was tested by immunoblot analysis. Anti-GBP did not react with either human or bovine fibronectin. Neither did anti-human and anti-bovine fibronectin react with GBP. Thus, GBP and mammalian fibronectins lack mutual immunological cross-reactivity. In addition, since no hemolymph proteins reacted with anti-human and anti-bovine fibronectin, the pearl oyster has no detectable level of immunologically relevant substances to mammalian fibronectins in the hemolymph.

Production of GBP by amebocytes

GBP in pearl oyster amebocytes was observed by indirect immunofluorescence. The agranular amebocytes exhibited a reaction to anti-GBP in the cytoplasm, as granular staining (Fig. 5a). On the other hand, the cells did not react with anti-collagen.

Next, we examined the location of GBP in amebocytes cultured for 6 days to determine

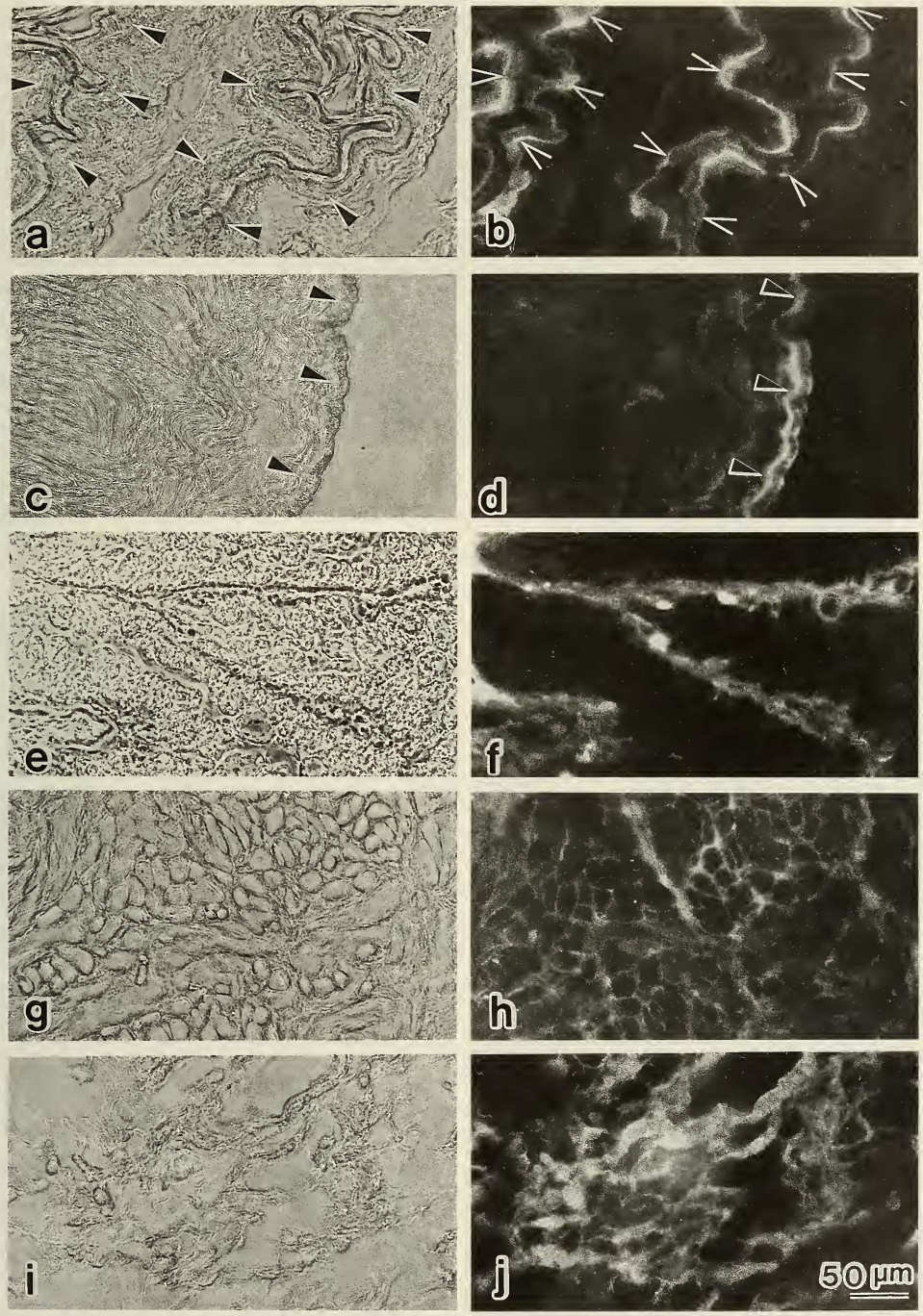
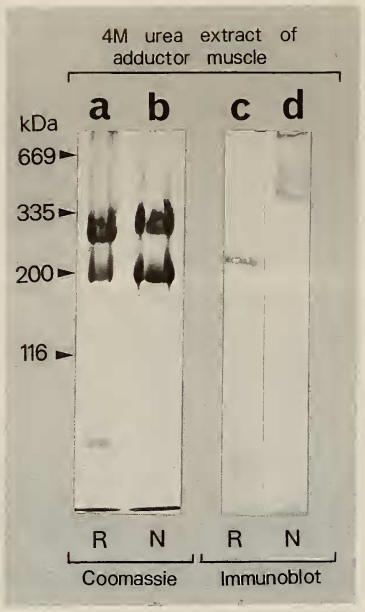
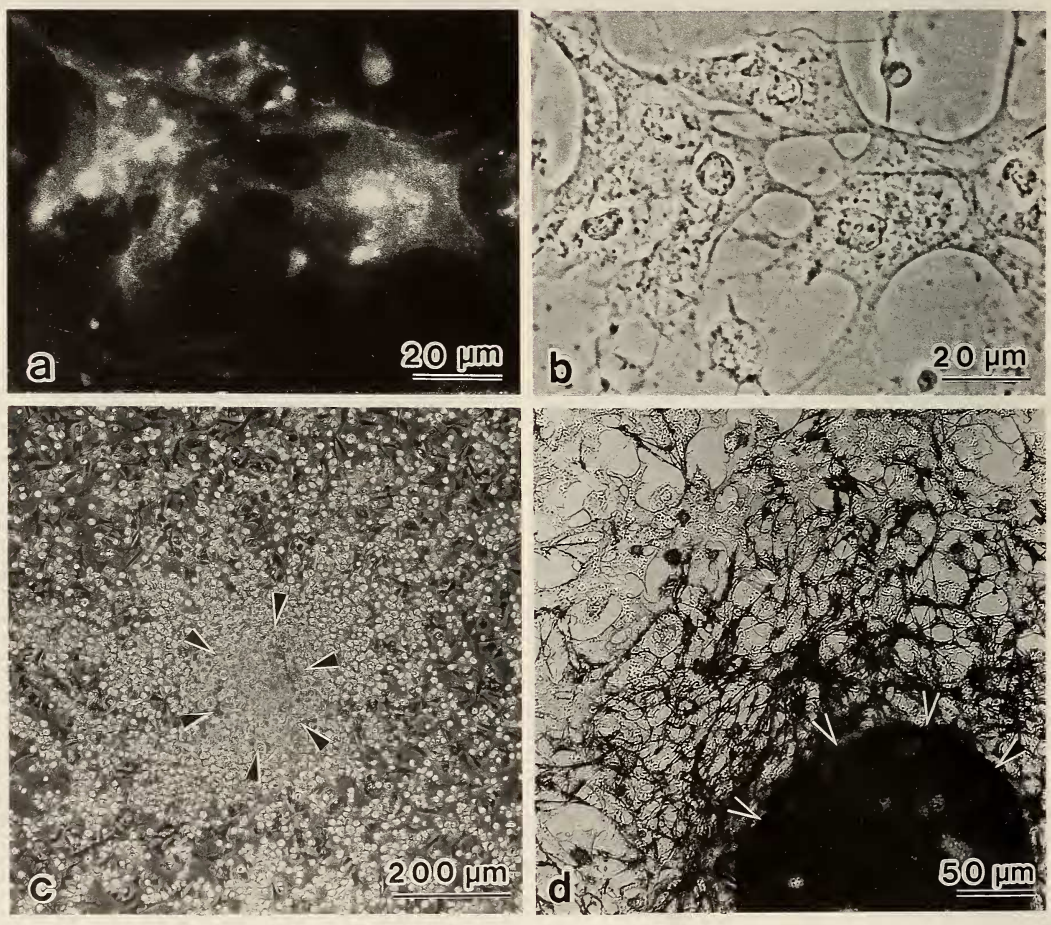


FIG. 3. Immunolocalization of GBP in the pearl oyster. The photographs on the right are of indirect immunofluorescence using anti-GBP. Those on the left are of phase contrast micrography of the same field as that on the right. a and b: byssus gland. The arrows indicate the basement membrane. c and d: body wall. The arrows show the basement membrane. e and f: kidney. g and h: muscle. i and j: loose connective tissue between digestive diverticula and body wall.



whether amebocytes secrete GBP into ECM. An ECM started to be deposited inside the aggregates of amebocytes after 3–4 days of culture, as reported by Suzuki et al. [5]. When enzyme-labeled staining was applied to 6-day cultured aggregates, the gel-like matrix in the aggregates displayed intense reaction with anti-GBP (Fig. 5d). Fibrillar staining was also detected around it. Thus, GBP was deposited *de novo* in the ECM secreted by amebocytes. In the control, using pre-immune serum, neither amebocytes nor the *in vitro* matrix

FIG. 4. Detection of GBP in pearl oyster adductor muscle of western blotting. 4 M urea extract of adductor muscle was electrophoresed in 5% polyacrylamide gel and subjected to protein staining or immunoblotting. a and b: Coomassie-stained gel. c and d: nitrocellulose sheet immunostained using anit-GBP. In each lane, 20 μg of protein was run. R and N, same as in Fig. 1.



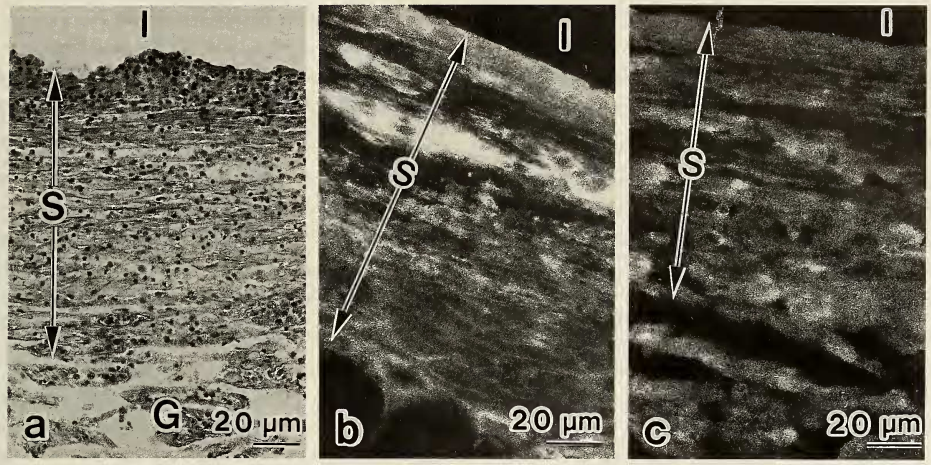


FIG. 6. Immunolocalization of GBP and type I-like collagen in the amebocyte sheath formed around an abiotic implant (shell bead) inserted into gonad (on day-7 after wounding). a: hematoxylin-eosin staining. b and c: indirect immunofluorescence using anti-GBP and anti-type I like collagen, respectively. G, gonad; I, abiotic implant; S, amebocyte sheath.

were stained.

Finally, GBP was localized at the experimental wound site. In this experiment, an abiotic implant (shell bead) was inserted into the gonad via an incision. At 7-day post wounding, the agranular amebocytes formed a cellular sheath, covering the implant (Fig. 6a). Reactions to anti-GBP and anti-collagen were detected in the amebocyte sheath (Fig. 6b, c).

DISCUSSION

Mammalian fibronectin exists in a soluble form in plasma and as an insoluble form in tissues and cell surfaces [28]. Plasma fibronectin has an approximate 450 kDa molecular mass and consists of two similar subunits of 220–240 kDa covalently bound by two disulfide bonds [29]. Plasma fibronectin is synthesized and secreted mainly by hepatocytes [30]. On the other hand, cellular fibronectin is synthesized by various cells such as fibroblasts, myoblasts and endothelial cells, and is highly polymerized as an insoluble form with other ECM components [31]. One major biological function of fibronectin is mediation of cell-adhesion.

In invertebrates, fibronectin(-like) molecules have been isolated and some of their biochemical properties have been described in coelenterates [19], sea-urchin *Pseudocentrotus depressus* [16, 32], snail *Helix aspersa* [17] and *Drosophila* [18]. Each type has the ability to bind to denatured mammalian collagen (i.e., gelatin). Tissue fibronectins of these animals structurally coincide well with mammalian fibronectin in both molecular weight and the possession of interchain disulfide bonds. However, the subunit structure of the soluble form of invertebrate fibronectins has not been described. Sea-urchin fibronectin promotes the spreading of BHK cells [32], so that the mediation of cell-adhesion seems to be a universal property of both vertebrate and invertebrate fibronectin(-like) molecules.

In this study, we fractioned hemolymph of the

FIG. 5. Immunolocalization of GBP in agranular amebocytes (a and b) and in an amebocyte aggregate depositing extracellular matrix (c and d). a: indirect immunofluorescence using anti-GBP. The cells were stained soon after blood collection. b: phase contrast micrography of the same field as that on a. c: phase contrast micrography of amebocyte aggregate cultured for 6 days. The extracellular matrix deposited inside the aggregate is shown by the arrows. d: enzyme-labeled antibody staining using anti-GBP showing that the matrix (arrows) is reactive.

pearl oyster, *Pinctada fucata*, by gelatin-affinity chromatography under conditions used for purifying mammalian plasma fibronectin [24]. This procedure yielded a monomeric gelatin-binding protein (GBP). GBP was similar in size to fibronectins with regard to the single polypeptide chain, but differed from it in subunit structure. Proteolysis of fibronectin yields a fragment almost as large as the original subunits when the first proteolytic cleavage occurs at the C-terminal end [33]. Among bivalves which have not developed a humoral clotting system [34], it is inconceivable that hemolymph proteins had rapidly degenerated under the purification conditions used here. However, it remains possible that the 220 kDa polypeptide is a product of proteolytic cleavage which may occur *in vivo*.

GBP was localized at the connective tissues of various organs using immunohistochemical techniques. Immunoblot analysis suggested the existence of an insoluble form of GBP in the tissues, which is polymerized by disulfide linkages. Thus, GBP seems to be present in the connective tissues as an insoluble component of the ECM.

GBP exhibited a low level of cell-spreading activity toward BHK cells compared with that of bovine fibronectin. One hypothesis is that the affinity of GBP for plasma membrane receptors of BHK cells is low due to the wide evolutionary distance between mammals and molluscs. For critical evaluation, GBP cell-spreading activity should be examined with cells of the pearl oyster. Therefore, we prepared primary cells (fibroblast-like) from several tissues of the pearl oyster, but, unfortunately, all cells rapidly spread in non-coated (control) plastic wells even in the physiological saline (data not shown).

In conclusion, GBP may be a cell-adhesive protein of the pearl oyster which exists in blood as a single 220 kDa polypeptide and also in tissue as polymerized insoluble form. GBP of the pearl oyster is a probable homologue (fibronectin-like molecule) to mammalian fibronectin, with which it shares some properties. Immunologically, GBP had no cross-reactivity with mammalian fibronectins. Since sea urchin and bovine fibronectins lack mutual cross-reactivity [16], immunological properties do not always serve as criteria for identifying fibronectin.

The molluscs have a wound healing system which widely differs from that of mammals [1–5]. In these animals, the wound site is healed via following four cellular reactions; the removal of tissue debris, cellular sheath formation, ECM production and epithelial regeneration. The cellular sheath formation is thought to be a hemostatic reaction in molluscs, which lack a humoral clotting system. In the pearl oyster, the agranular amebocytes, which are macrophage-like cells, are responsible for not only phagocytosis of debris but also the two successive healing reactions [5]. The epithelial cells migrate along the ECM newly secreted by the sheath amebocytes [5].

The agranular amebocytes have ability to secrete type I-like collagen and proteoglycans as components of the ECM, *in vitro* [5]. The present study demonstrated that GBP exists in the ECM produced by the amebocytes, suggesting that it is secreted with other ECM components by the cells. At the experimental wound site in the gonad, where an abiotic implant (shell bead) was inserted via an incision, the agranular amebocytes formed a sheath of 10–20 cell layers to cover the implant, after which the ECM began to be deposited in the spaces between sheath cells [5]. It was ascertained that GBP is deposited with type I-like collagen in the amebocyte sheath. These data suggest that GBP is synthesized and secreted by the agranular amebocytes with other ECM components during wound healing. It is hypothesized that GBP acts as a mediator of cell-adhesion for migrating epithelial cells at wound sites in the pearl oyster.

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REFERENCES

1 DesVoigne, D. M. and Spark, A. K. (1968) The process of wound healing in the Pacific oyster, *Crassostrea gigas*. J. Invert. Pathol., **12**: 53–65.

2 Pauley, G. B. and Heaton, L. H. (1969) Experimental wound repair in the freshwater mussel

Anodonta oregonensis. J. Invert. Pathol., **13**: 241–249.

3 Ruddell, C. L. (1971) The fine structure of oyster agranular amebocytes from regenerating mantle wounds in the Pacific oyster, *Crassostrea gigas*.J. Invert. Pathol., **18**: 260–268.

4 Sminia, T., Pietersma, K. and Scheerboom, J. E. M. (1973) Histological and ultrastructural observations on wound healing in the freshwater pulmonate *Lymnaea stagnalis*. Z. Zellforsh., **141**: 561–573.

5 Suzuki, T., Yoshinaka, R., Mizuta, S., Funakoshi, S. and Wada, K. (1991) Extracellular matrix production by amebocytes during epithelial regeneration in the marine bivalve *Pinctada fucata*. Cell Tissue Res., **266**: 75–82.

6 Bohn, H. (1975) Growth-promoting effect of haemocytes on insect epidermis *in vitro*. J. Insect Physiol., **21**: 1283–1293.

7 Bohn, H. (1977a) Conditioning of a glass surface for the outgrowth of insect epidermis (*Leucophaea maderae*, Blattaria). In Vitro, **13**: 100–107.

8 Bohn, H. (1977b) Enzymatic and immunological characterization of the conditioning factor for epidermal outgrowth in the cockroach *Leucophaea maderae*. J. Insect Physiol., **23**: 1063–1073.

9 Clark, R. A. F., Lanigan, J. M., DellaPelle, P., Manseau, E., Dvorak, H. F. and Colvin, R. B. (1982) Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization. J. Invest. Dermatol., **79**: 264–269.

10 Fujikawa, L. S., Foster, C. S., Gipson, I. K. and Colvin, R. B. (1984) Basement membrane components in healing rabbit corneal epithelial wounds: immunofluorescence and ultrastructural studies. J. Cell. Biol., **98**: 128–138.

11 Yamada, K. M. (1988) Fibronectin domains and receptors. In "Fibronectin." Ed. by D. F. Mosher, Academic Press, San Diego, pp. 48–121.

12 Bronner-Fraser, M. (1986) An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development. Dev. Biol., **117**: 528–536.

13 Boucaut, J. C., Darribere, T., Boulekbache, H. and Thiery, J. P. (1984) Prevention of gastrulation but not neurulation by antibodies to fibronectin in amphibian embryos. Nature, **307**: 364–367.

14 Thiery, J. P., Duband, J. L. and Delouvee, A. (1982) Pathways and mechanism of avian trunk neural crest cell migration and localization. Dev. Biol., **93**: 324–343.

15 Akiyama, S. K. and Johnson, M. D. (1983) Fibronectin in evolution: presence in invertebrates and isolation from *Microciona prolifera*. Comp. Biochem. Physiol., **76B**: 687–694.

16 Iwata, M. and Nakano, E. (1981). Fibronectin from the ovary of the sea urchin, *Pseudocentrotus depressus*. Wilhelm Roux's Archives., **190**: 83–86.

17 Bride, M., Barre, P., Griffond, B. and Bride, J. (1988) Localization of a fibronectin-like molecule in the ovotestis of the snail *Helix aspersa*. Cell Tissue Res. **254**: 421–428.

18 Gratecos, D., Naidet, C., Astier, M., Thiery, J. P. and Semeriva, M. (1988) *Drosophila* fibronectin: a protein that shares properties similar to those of its mammalian homologue. EMBO J., **7**: 215–223.

19 Schlage, W. K. (1988) Isolation and characterization of a fibronectin from marine coelenterates. Eur. J. Cell Biol., **47**: 395–403.

20 Labat-Rabert, J., Robert, L., Auger, C., Lethias, C. and Garrone, R. (1981) Fibronectin-like protein in Porifera: its role in cell aggregation. Proc. Natl. Acad. Sci. USA., **78**: 6261–6265.

21 Katow, H., Yamada, K. M. and Solursh, M. (1982) Occurrence of fibronectin on the primary mesenchyme cell surface during migration in the sea urchin embryo. Differentiation, **22**: 120–124.

22 Katow, H., Yazawa, S. and Sofuku, S. (1990) A fibronectin-related synthetic peptide, Pro-Ala-Ser-Ser, inhibits fibronectin binding to the cell surface, fibronectin-promoted cell migration *in vitro*, and cell migration *in vivo*. Exp. Cell Res., **190**: 17–24.

23 Suzuki, T. and Mori, K. (1989) A galactose-specific lectin from the hemolymph of the pearl oyster, *Pinctada fucata martensii*.Comp. Biochem. Physiol., **92B**: 455–462.

24 Ruoslahti, E., Hayman, E. G., Pierschbacher, M. and Engvall, E. (1982) Fibronectin: purification, immunochemical properties, and biological activities. Methods. Enzymol., **82**: 803–831.

25 Isemura, M., Yamaguchi, Y., Munakata, H., Aikawa, J., Kan, M., Yamane, I. and Yosizawa, Z. (1984) Isolation and characterization of human placenta fibronectin. J. Biochem., **96**: 163–169.

26 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**: 680–685.

27 Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA., **76**: 4350–4354.

28 Yamada, K. M., Hayashi, M., Hirano, H. and Akiyama, S. K. (1984) Fibronectin and cell surface interactions. In "The role of extracellular matrix in development." Ed, by D. F. Mosher, Alan R. Liss, New York, pp. 89–121.

29 Kornblihtt, A. R. and Gutman, A. (1988) Molecular biology of the extracellular matrix proteins. Biol. Rev., **63**: 465–507.

30 Tamkun, J. W. and Hynes, R. O. (1983) Plasma fibronectin is synthesized and secreted by hepatocytes. J. Biol. Chem., **258**: 4641–4647.

31 Hynes, R. O. and Yamada, K. M. (1982) Fibronectin: multifunctional modular glycoproteins. J. Cell. Biol., **95**: 369–377.

32 Iwata, M. and Nakano, E. (1983) Characterization of sea-urchin fibronectin. Biochem. J., **215**: 205–208.

33 Mosesson, M. W., Chen, A. B. and Huseby, R. M. (1975) The cold-insoluble globulin of human plasma: studies of its essential structural features. Biochim. Biophys. Acta, **386**: 509–524.

34 Needham, A. E. (1970) Haemostatic mechanisms in the invertebrates. Symp. Zool. Soc. Lond., **27**: 19–44.